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<p>13. Abstract (<i>Maximum 200 Words</i>) (<i>abstract should contain no proprietary or confidential information</i>)</p> <p>We have isolated a human cDNA clone containing an open reading frame (ORF) for a protein that has amino acid sequence similarity to an FGF-binding protein (FGF-BP1; M60047). The gene structure of human FGF-BP1 is similar to that of mouse FGF-BP1 previously described by our group. Deduced amino acid sequence of FGF-BP2 reveals that the protein is composed of 223 amino acid residues with a M_r of 24.5 kDa, including a putative 19 amino acid signal peptide. It shows 21% amino acid identity and 41% homology to FGF-BP1. To investigate the function of FGF-BP2, a human adrenal carcinoma SW-13 cell line was used as a model system. We found that FGF-BP2 is secreted as a heparin-binding protein in the culture media of stably transfected cells, and capable of interacting with ¹²⁵I-FGF-2. FGF-BP2 promotes colony formation in soft agar and enhances activities of added bFGF and aFGF. Overexpression induces tumor growth of SW-13 cells and increases tumor incidence of MCF-7 cells in athymic nude mice. Furthermore, abundant FGF-BP2 mRNA was expressed in breast cancer primary and metastatic tumor tissues by <i>in situ</i> hybridization, suggesting that the molecule may play a role in breast cancer growth and metastasis.</p>					
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Introduction

The fibroblast growth factor (FGF) family comprises of at least 20 genetically distinct gene members which play an important role in embryonic developmental processes, in angiogenesis, wound healing, tumor growth, and in neuronal function and neuronal regeneration. *In vitro*, they are potent regulators of proliferation, differentiation, motility and survival for various cell lines (Reviews in 1,2). The prototypic members of this family are the acidic and basic FGF (FGF-1 and FGF-2, respectively) that are widely expressed and distributed in the extracellular matrix of embryonic, adult and tumor tissues (3,4). In the extracellular matrix, these fibroblast growth factors are bound to matrix heparin sulfate proteoglycans and remain in a latent state until they are activated and mobilized to their high affinity tyrosine kinase receptors to elicit their biological activities. One established mechanism that can release active FGFs from the extracellular storage site is the digestion of heparin sulfate chains and proteoglycan protein cores by extracellular heparanases and proteinases, respectively (5,6). Recently, we proposed an alternative mechanism that involves a secreted FGF-binding protein, FGF-BP1 (7,8,9).

FGF-BP1 is a secreted heparin binding protein that binds to FGF-1 and FGF-2 in a non-covalent, reversible manner (7). FGF-BP1 mRNA is expressed at high levels in squamous cell carcinoma (SSC) tissues, SSC cell lines of different origin, and in some colon cancer cell lines (8,9). We have shown that overexpression of FGF-BP1 in a non-tumorigenic human cell line SW-13 that expresses FGF-2, induces these cells to grow colonies in soft agar, and to form highly vascularized tumors in athymic nude mice (8). When endogenous FGF-BP1 levels of human SSC (ME-180) and colon carcinoma (LS174T) cell lines were depleted by specific targeting ribozymes, we found that the reduction of FGF-BP1 decreased the release of biologically active FGF-2 from cells in culture (9). In addition, the growth and angiogenesis of xenograft tumors in mice were decreased in parallel with the reduction of FGF-BP1. Thus, our studies suggest that FGF-BP1 can mobilize and activate the latent FGF-2 stored in the matrix, and being utilized as an angiogenic switch molecule in some human tumors.

Recently, we have isolated a human cDNA clone containing an open reading frame (ORF) for a protein that has amino acid sequence similarity to FGF-BP1. In this study, we examined the structure and function of this novel FGF-BP molecule that we named FGF-BP2. We show that FGF-BP2 is also able to mobilize, activate latent FGFs, and to induce tumors in athymic nude mice. Furthermore, the distinct expression pattern of this molecule in clinical breast cancer samples suggests that FGF-BP2 may play an important role in tumor growth and metastasis.

Body

Genomic sequence analysis of FGF-BPs

Sequence similarity searches revealed that cDNA sequences of both human FGF-BP1 and FGF-BP2 are located on one of the six unordered genomic DNA pieces derived from a BAC clone C0024K08 for human chromosome 4 whose genome sequencing is in progress (Accession number AC005598). On the basis of the human FGF-BP1 cDNA sequence published by Satos group (7) and the FGF-BP1 promoter sequence isolated in our laboratory (10), the FGF-BP1 gene consists of two exons separated by an intervening intron of 1.6 kb in length as depicted in Fig. 1a. The entire ORF of FGF-BP1 is located on exon 2, whereas exon 1 contains most of the 5' untranslated region of the gene. The gene structure of human FGF-BP1 is very similar to that of mouse FGF-BP1 previously described by our group (Fig. 1b and c) (11). About 22 kb 5' upstream of FGF-BP1 transcription start site lies the most 3' end of FGF-BP2 gene (Fig. 1a). Although the promoter region of FGF-BP2 has not been determined, on the basis of its cDNA sequence and the most 5' expressed sequence tag containing a part of the FGF-BP2 coding region (Accession number AA317400), it appears that the gene is also composed of 2 exons with an intervening intron of 1.8 kb in length (Fig. 1a). However, unlike the FGF-BP1 gene, exon 1 contains entirely the ORF of FGF-BP2. On this genomic contig, there are additional sequences of about 34 kb and 30 kb on the 5' and 3' ends of FGF-BP2 and FGF-BP1 genes, respectively. Using GeneMap of the human genome, both human FGF-BP1 and FGF-BP2 genes are found to reside on the short arm of human chromosome 4 between D4S412 and D4S1601 (3.7 - 28.2 cM) microsatellite anchor markers.

Sequence analysis of FGF-BP2 cDNA

Our isolated full length FGF-BP2 cDNA contains 1120 base pairs excluding the poly-(A) tail, and its sequence is mapped between nucleotides at position 132,636 and 135,582 on a genomic contig of the BAC clone described above. The ORF extends from an ATG start codon at nucleotide 64 to a TGA stop codon at nucleotide 736. There is no homology between FGF-BP1 and FGF-BP2 cDNA sequences. The deduced amino acid sequence of FGF-BP2 contains 223 amino acids with a calculated M_r of 24.5 kDa and a pI of 9.15. Using a computer program predicting prokaryotic and eukaryotic signal peptides and their cleavage sites (12), the signal peptide for FGF-BP2 would consist of the first 19 amino acid residues with the potential cleavage site occurs between G₁₉ - Q₂₀ (Fig. 1c, arrow). Deduced amino acid sequences of FGF-BP1 and FGF-BP2 are aligned for comparison (Fig. 1c). Overall, FGF-BP2 has 21% amino acid identity,

and 41% homology with respect to FGF-BP1. In contrast, human FGF-BP1 shows 63% amino acid identity and 74% homology to mouse FGF-BP1 (11). Both FGF-BPs contain 8 conserved cysteine residues indicated by stars in Fig. 1c, suggesting these residues are probably critical for the function of the proteins.

Biochemical characterization of recombinant human FGF-BP2.

To demonstrate that FGF-BP2 is capable of binding FGF-2 and heparin, we have constructed and expressed FGF-BP2 containing a Myc/His tag at its carboxyl terminus. Proteins in serum-free culture media of FGF-BP2/Myc/His or mock transfectants were incubated with Ni-NTA resins, then subjected to immunoblotting analysis using a monoclonal antibody specific for the His tag. A reactive band of M_r 38 kDa was only observed for samples of FGF-BP2/Myc/His transfected cells (Fig. 2a). A similar reactive band was detected on the blots that were probed separately with polyclonal antibodies raised against FGF-BP2 peptides (data not shown). Upon incubation of the samples with Ni-NTA resins in the presence of ^{125}I -FGF-2, in addition to the immunoreactive band, a strong radiolabeled band of M_r 17 kDa was detected in samples of FGF-BP2 transfectants; whereas a faint radioactive band was observed for mock control samples (Fig. 2a). These data demonstrate that FGF-BP2 can interact with FGF-2. An immunoreactive band of M_r 38 kDa was also detected for FGF-BP2/Myc/His samples eluted from heparin-Sepharose beads, indicating that FGF-BP2 is a heparin binding protein (data not shown).

Expression and biological activity of FGF-BP2 in stable SW-13 transfectants

To examine the potential role of FGF-BP2 in tumor growth, a human adrenal carcinoma SW-13 cell line was selected as a model system. This cell line expresses high levels of FGF-2, but lacks both FGF-BP1 and FGF-BP2 expression (8). Furthermore, wild type SW-13 cells do not form colony in soft agar, nor do they form tumors in athymic nude mice unless they are supplemented with exogenous FGFs or transfected with FGF genes with secreted signal peptides (13).

We transfected SW-13 cells with a eukaryotic pCR3.1 vector containing an ORF of FGF-BP2 whose expression is driven by the CMV promoter. Cells were also transfected with an empty vector as a negative control and stably transfected cells were selected after G418 treatment. Northern blotting analysis revealed a high FGF-BP2 mRNA level in FGF-BP2 transfectants, whereas no signal was detected in mock-transfected cells (data not shown).

Transfected cells were plated on agarose dishes. There were twice as many colonies formed in soft agar by FGF-BP2 transfectants compared to the mock-transfected cells (Fig. 2b), indicating that FGF-BP2 promotes colony formation. Both transfected cell populations were stimulated to form more colonies in the presence of exogenous FGF-2. However, we consistently observed twice as many colonies formed by FGF-BP2 transfectants relative to control cells at low exogenous FGF-2 concentrations (0.05 - 0.10 ng/ml). At high exogenous FGF-2 concentrations (> 0.25 ng/ml), the difference observed for the two cell populations is greatly diminished. It appears that FGF-BP2 enhances the availability of active FGF-2 at low concentrations to cellular receptors, whereas at high concentrations the receptors are probably all saturated. In the presence of 1ng/ml FGF-2, treatment with an anti-FGF-2 antibody (1 μ g/ml) effectively reduced colony formation in FGF-BP2 transfectants to the same level as that of untreated mock transfected cells, indicating that free FGF-2 is the driving force for colony formation (data not shown). Similar observations were made when exogenous FGF-1 was used instead of FGF-2, although relatively high FGF-1 concentration levels were required. Treatment with an anti-FGF-2 antibody (1 μ g/ml) had no apparent effect on colony formation for FGF-BP2 transfectants in the presence of 0.1ng/ml of FGF-1, indicating the specificity of the antibody used.

FGF-BP2 transfected SW-13 cells were injected subcutaneously into athymic nude mice and tumor growth was examined over an 8 week period. Mock-transfected SW-13 cells were used as a negative control. Tumors were found in all 3 animals injected with FGF-BP2 transfected cells while animals injected with mock-transfected cells were tumor free, indicating that FGF-BP2 supports tumor growth *in vivo* (Fig. 2c). High standard deviations were observed for the means of tumor size, indicating biological differences in the initiation and growth of tumors in different animals. Northern blotting analysis revealed that cells in all excised tumors expressed high mRNA levels of FGF-BP2.

FGF-BP2 regulates tumor size and incidence for MCF-7 breast cancer cells

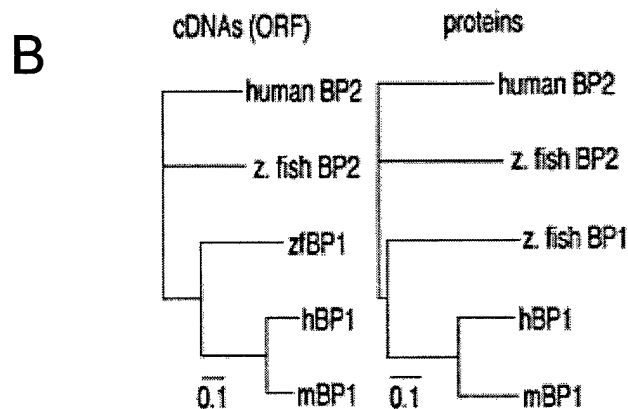
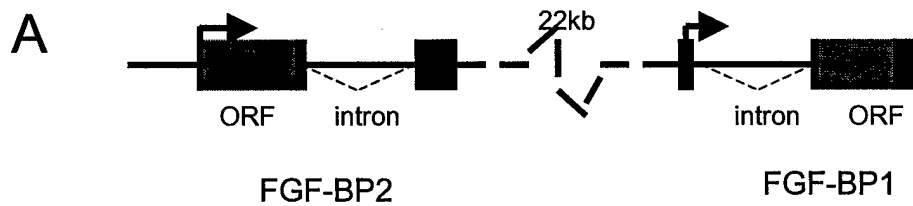
To examine the role of FGF-BP2 in a breast cancer cell model, FGF-BP2 was expressed in MCF-7 human breast cancer cells. Cells were implanted into the mammary fat pad of athymic nude mice and tumor incidence as well as tumor growth was monitored over a period of three weeks. We observed that both tumor incidence and tumor size was affected by the overexpression of FGF-BP2. We observed that BP2 expression greatly increases tumor incidence in 3 independent experiments. For the data shown in figure 3a, the p-value is based on a chi-

square analysis. $P = 0.0043$ suggests a highly significant difference between the BP2 and the control group with respect to the ability of the tumors to take after the inoculation. Furthermore we observed that FGF-BP2 also affected the size of those tumors that did grow. The data from one representative experiment out of three are shown (Fig. 3b). The tumor sizes at week two after implantation (mean \pm SEM) are shown. The number of tumors growing in the BP2 and control groups were $n=9$ and $n=5$ respectively. The p-value is derived from a t-test and indicates a statistically significant difference amongst the groups.

FGF-BP2 in clinical breast cancer samples

In situ hybridization was used to detect BP2 expression in non-cancerous breast tissues, in in-situ ductal and lobular carcinoma (DCIS/LCIS) and in invasive breast cancers. The panels in figure 4 show *in situ* hybridization of paraffin-embedded sections for FGF-BP2 mRNA. Digoxigenin-labelled antisense riboprobes were used as positive and sense as negative control riboprobes. The total number of samples analyzed and the statistical comparison between non-cancerous, in situ carcinoma and invasive cancer is given in Table 1. Chi-square analysis with the Prism Instat statistics program was carried out using a correlation analysis without the liver metastases data, since we only had only two different cases available. A highly significant difference amongst columns (positive or negative for BP2) with a $p = 0.0038$ was found. This means that BP expression distinguishes amongst the different groups. In addition, a highly significant p-value for a correlation trend amongst rows and columns was found: $p = 0.0009$ for trend. Since the data were arranged to represent progression from normal via in situ carcinoma to invasive cancer, this trend supports the notion that BP2 expression increases with malignant progression.

Figure 1



C

murineBP1	----MRLHSLILLSFLLLATQAFSEKVRKRAKNAPHSTAEEGVEGSAPSLGKAQNKQRSR
ratBP1	----MRIHGLILLSFLLLAQVLSEKVRKTAKNVDPSTTEEDMS--PSLGKARNKQRSR
humanBP1	----MKICSLTLLSFLLLAQVLLVEGKKKVKNGLHSHKVVSEQK--DTLGNTQIKQKSR
humanBP2	----MKFPVPCLLLVTLSCGLTLCQAPRQKQGST-----GERPH-----
murineBP1	TSKSLTHGKFPVTKDQ-ATCRWAVTEEEQG---ISLKVQCT-QADQEFSCVFAGDPTDCLK
ratBP1	TSKSMTHGRFPVTKDQ-ATCRWAVTEEEELG---INLKVQCT-RADQEFSCVFAGDPTGCLK
humanBP1	PGN---KGKFPVTKDQ-ANCRWAATEQEEG---ISLKVECT-QLDHEFSCVFAGNPTSCLK
humanBP2	-----PQTGGRD---SCTNRPSLSLQCGAGEVWLRVDCR-NTDQTYWCEYRGQPSMCQA
	* * * *
murineBP1	HDKD-QIYWKQVARTLRKQKNICRDAKSVLKTRVCRKFPBSNLKLVNPNARG--NTKPR
ratBP1	YDKD-QTYWKQVARTLRKQKNICENSKSVLKTRVCRKFPBSNLKVNNP-----R
humanBP1	LKDE-RVYWKQVARNLRSQKDCRYSKTAVKTRVCRKDFPBSSSLKLVSTLFG--NTKPR
humanBP2	FAADPKSYWNQALQELRRLHHACQGA-PVLRPSVCREAGPQAHMQQVTSCLKGSPEPNQQ
	* *
murineBP1	KEKAEVSAAREHNKVQEAVSTEPNR--IKEDITLNPAAQTMTIRDPECLEDDVVLNQRT
ratBP1	KEKAEVSPREHNKVQEAVSMEPNK--VKVDITTSPAAT--VAVKDSECLEDDVVLNQRT
humanBP1	KEKTEMSPREHIKGETTPS-----SLAVTQTMATKAPECVEDPDMANQRT
humanBP2	PEAGTPSLRPKATVKLTATQLGKDSMEBLGKAKPTTRPTAKPTQPGPRPG-GNBEAKKK
murineBP1	ALEFCGESWSSICTFFLNMLQATSC
ratBP1	ALEFCGESWSSFCTFFLNMLQATSC
humanBP1	ALEFCGETWSSLCTFFLSIVQDTSC
humanBP2	AWEHCWKPFQALCAPLISFPRG
	* *

Figure 2

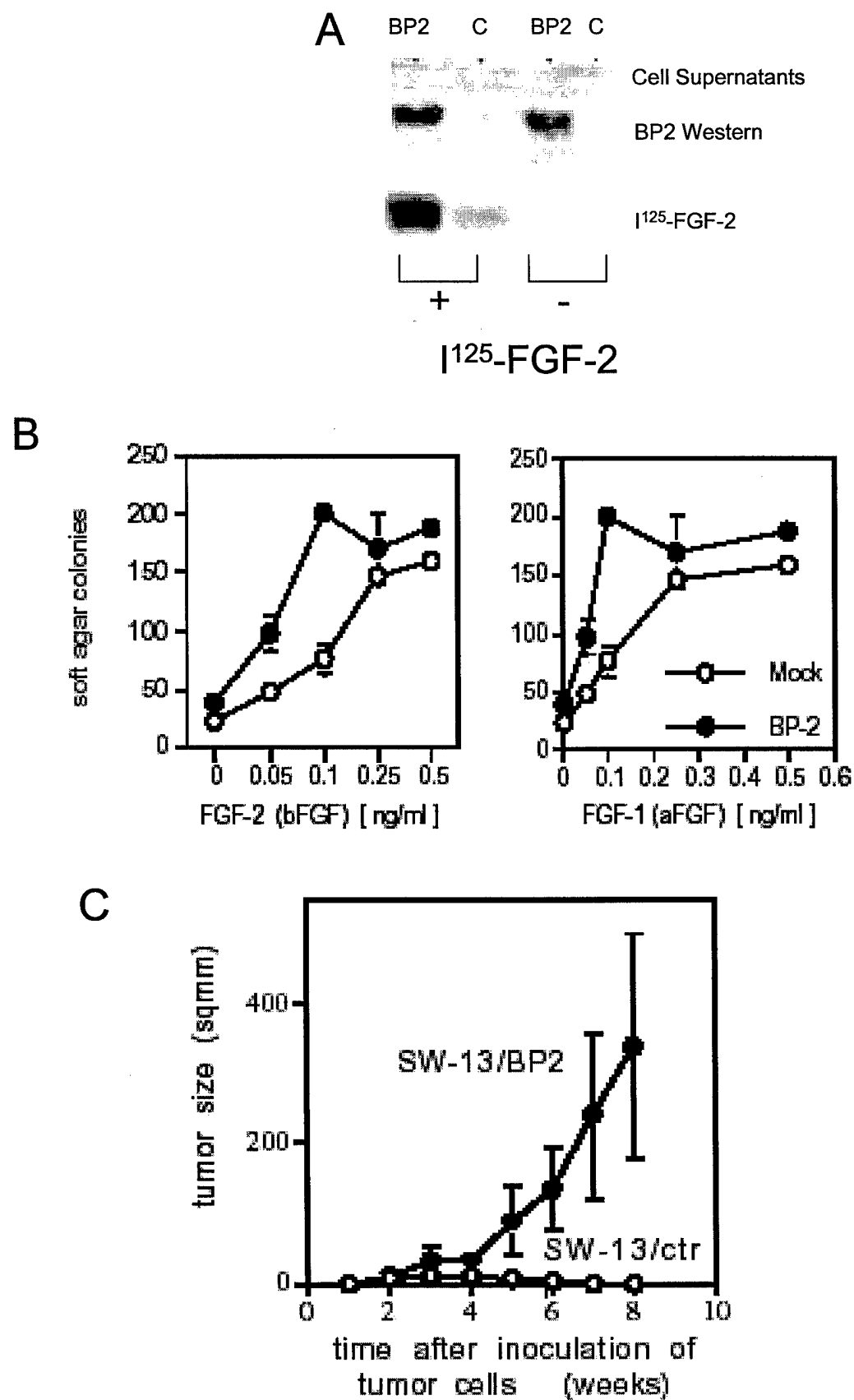


Figure 3

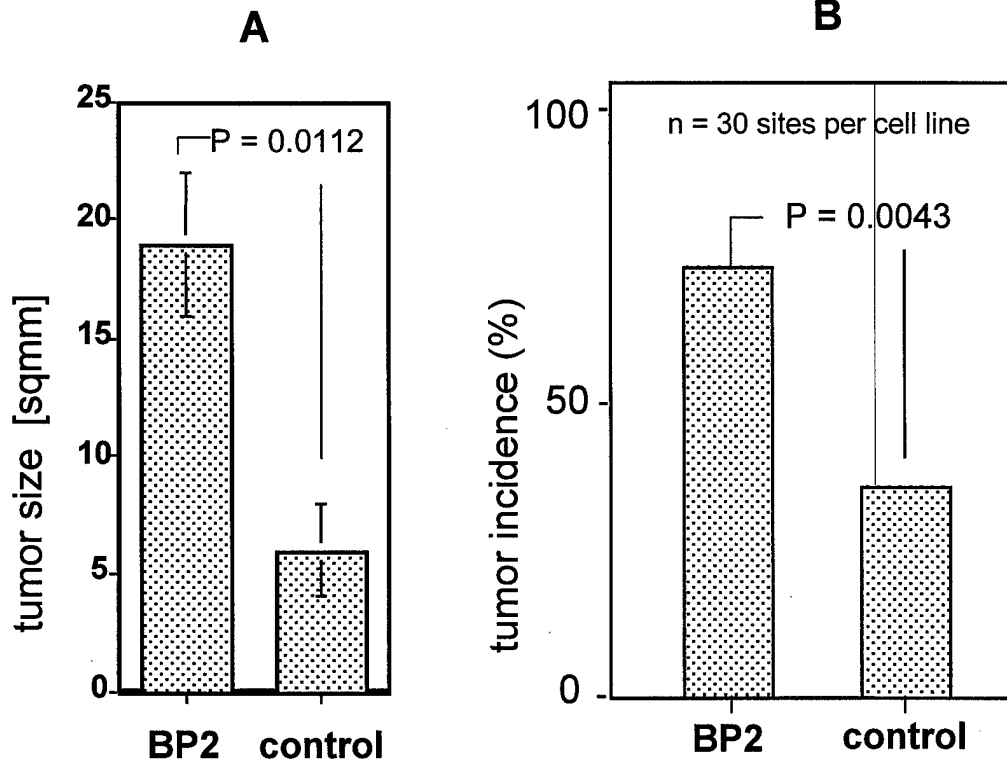


Figure 4

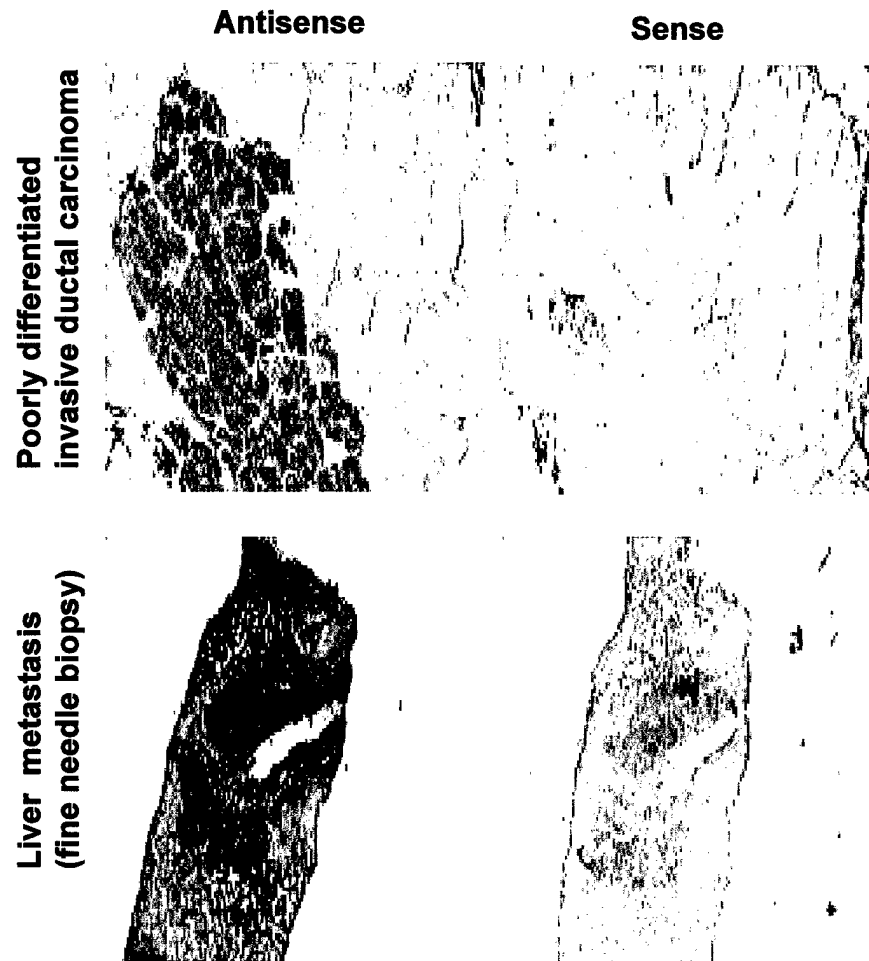


Table 1 BP2 mRNA expression analysis in breast tissues.

	BP-2 expression:	+	-
non-cancerous breast tissue	n=	4	13
in situ carcinoma (DCIS/LCIS)	n=	11	9
invasive carcinoma	n=	17	5
liver metastases	n=	2	0

Figure Legends

Figure 1. Analysis of the FGF-BP2 Gene. **A.** Gene structure of FGF-BP1 and BP2. **B.** Gene Tree analysis of human, murine and zebra fish BP1 and BP2 cDNA (ORF) and protein. The length of the lines relative to the scale bar gives the relative divergence amongst the sequences (Gene Tree Program from: r.page@bio.gla.ac.uk; web site: <http://taxonomy.zoology.gla.ac.uk>). **C.** Amino acid alignment of murine, rat and human FGF-BP1 with human BP2.

Figure 2. FGF-BP2 binds to FGF and exhibits a biological effect. **A.** Pull-down of [I-125]-FGF-2 by his-tagged BP2. Supernatants from SW-13 cells transfected with a poly-histidine-tagged BP2-protein expression vector were reacted with radiolabelled FGF-2. Supernatants from empty vector transfected SW-13 cells were used as a negative control. BP2 protein was pulled down by nickel-affinity beads and unbound proteins (and FGF-2) washed off, separated by SDS-PAGE and detected by Western blotting (= BP2; top panel) or by autoradiography (= FGF-2; bottom panel). **B.** BP2 effects in vitro SW-13 cells stably transfected with BP2 (SW-13/BP2 cells) were tested for their soft agar colony formation and response to different concentrations of added FGF-1 (aFGF) or FGF-2 (bFGF). No heparin was added to this assay. **C.** BP2 effects in vivo SW-13 cells stably transfected with BP2 (SW-13/BP2 cells) were tested for tumor growth as xenografts in athymic nude mice. Tumor growth in animals of SW-13/BP2 cells (n=3 mice per data point).

Figure 3. Effect of FGF-BP2 expression on tumor growth of human breast cancer cells. FGF-BP2 was expressed in MCF-7 human breast cancer cells. Cells were implanted into the mammary fat pad of athymic nude mice and tumor incidence as well as tumor growth was monitored. **A.** The impact of BP2 expression on tumor incidence out of a total of 30 implanted sites for each of the two groups in 3 independent experiments. The p-value is based on a chi-square analysis. $P = 0.0043$ suggests a highly significant difference between the BP2 and the control group with respect to the ability of the tumors to take after the inoculation. **B.** The impact of BP2 expression on the size of tumors grown. The data from one representative experiment out of three are shown. The tumor sizes at week two after implantation (mean \pm SEM) are shown. The number of tumors growing in the BP2 and control groups were n=9 and n=5 respectively. The p-value is derived from a t-test and indicates a statistically significant difference amongst the groups.

Figure 4. Expression of FGF-BP2 in breast cancer and Table 1 BP2 mRNA expression analysis in breast tissues. The panels show *in situ* hybridization of paraffin-embedded sections for FGF-BP2 mRNA. Digoxigenin-labelled antisense riboprobes were used as positive and sense as negative control riboprobes. The total number of samples analyzed and the statistical comparison between non-cancerous, in situ carcinoma and invasive cancer is given in Table 1. *In situ* hybridization was used to detect BP2 expression in non-cancerous breast tissues, in in-situ ductal and lobular carcinoma (DCIS/LCIS) and in invasive breast cancers. Chi-square analysis with the Prism Instat statistics program was carried out using a correlation analysis without the liver metastases data, since we only had two different cases available. A highly significant difference amongst columns (positive or negative for BP2) with a $p=0.0038$ was found. This means that BP expression distinguishes amongst the different groups. In addition, a highly significant p-value for a correlation trend amongst rows and columns was found: $p=0.0009$ for trend. Since the data were arranged to represent progression from normal via in situ carcinoma to invasive cancer, this trend supports the notion that BP2 expression increases with malignant progression

Key Research Accomplishments

During the course of these studies, we have elucidated that:

- The gene structure of FGF-BP2 is delineated on the basis of sequencing information available in the database.
- FGF-BP2 has tumor promoting activity in vitro and in vivo. Its activity is presumably mediated through the solubilization of extracellular matrix bound FGFs.
- FGF-BP2 is a heparin-binding, secreted protein and it is capable of interacting with FGF-2.
- The expression pattern of FGF-BP2 is distinct from that of FGF-BP1. Abundant FGF-BP2 messages are found to be up-regulated in melanoma and breast tumor tissues.
- Overexpression of FGF-BP2 can contribute towards hormone independent tumor growth
- There is a direct correlation between tumor progression and FGF-BP2 expression
- Inhibition of FGF-BP2 may prevent tumor progression

Reportable Outcomes

- Individual Allocation Grant from American Cancer Society. A \$15,000, non-renewable grant was recently obtained for the period July 1999 to June 2000, to work on the FGF-BP2 project. The grant covers the reagent supplies for the project.
- A manuscript based on the work of FGF-BP2 is ready for submission. When it is accepted in a peer-reviewed journal, a copy will be forwarded. "Mobilization of latent FGFs and induction of tumor growth by a novel secreted FGF-binding protein (FGF-BP2)"
- Work was presented at the Department of Defense Breast Cancer Research Program Era of Hope Meeting, June 2000. "Mobilization of latent FGFs and induction of tumor growth by a novel secreted FGF-binding protein (FGF-BP2)"

Conclusions

In this study, we have examined the gene structure and function of a novel secreted fibroblast growth factor-binding protein, FGF-BP2. Performing sequence similarity searches, we are able to map both cDNA sequences of human FGF-BP1 and FGF-BP2 on a chromosome 4 genomic fragment of 91.5 kb in length. On the basis of this genomic sequence and other related sequences available in the GenBank data base, the gene structures for both FGF-BPs are deduced as shown in Fig. 1. Since the promoter region of FGF-BP2 has not been defined, we can not preclude the possibility that FGF-BP2 gene may contain another exon encoding for its 5'-untranslated region. Apparently, the 200 bp region immediately 5' upstream of the putative transcription start site of the FGF-2 gene does not contain any TATA box or homologous regulatory elements such as: Ets, AP-1, SP1 and CAATT/enhancer binding protein C/ERP sites as those found in human FGF-BP1 promoter (10). This suggests that these FGF-BP genes are probably regulated by different mechanisms. The distinct mRNA expression patterns observed for these FGF-BPs in different tissues, cell lines of different origins seem to support this argument. Furthermore, our preliminary data indicate that retinoids and phorbol esters, known modulators of FGF-BP1 expression (10, 15), have little apparent effect on the constitutive message levels of FGF-BP2 in melanoma cells (data not shown).

The deduced amino acid sequence of FGF-BP2 suggests a secreted protein with a M_r of about 22 kDa without the putative signal peptide. Surprisingly, we observed in the supernatant of transfected cells predominantly a 38 kDa protein that is reactive to anti-His/Myc tag monoclonal antibodies and anti-FGF-BP2 peptide polyclonal antibodies. *In vitro* coupled transcription/translation reactions of the expression plasmid containing the ORF of FGF-BP2 also yielded a radioactive band of similar molecular weight (data not shown). Based on the apparent molecular weight difference, we assume that this is the dimeric form of FGF-BP2. Interestingly, this association of FGF-BP2 monomers is covalently linked, and not mediated through cysteine residues. We are currently investigating further the biochemical nature of this dimer, and determine whether the dimeric form of FGF-BP2 is essential for FGF-2 interaction and for its tumor promoting properties.

Similar to the function previously ascribed to FGF-BP1 (8, 9), soft agar data suggest that FGF-2 is able to activate endogenous matrix-bound FGF-2 and allows it to reach its cellular receptors in the SW-13 model system. In addition, we also showed that FGF-BP2 is effective in presenting low concentrations of both exogenous FGF-1 and FGF-2 to their receptors to induce colony formation. Similar mobilization of FGF-2 deposited in the matrix by tumor cells and/or

host stromal cells by FGF-BP2 secreted by SW-13 cells enables tumors formed in nude mice injected with FGF-BP2 SW-13 transfectants. Our data is compatible with a model in which FGF-BP2 displaces FGF-2 bound to matrix heparan sulfate proteoglycans and presents it to its receptors. However, it is further complicated by the ability of FGF-BP2 to bind to heparin-Sepharose, and therefore likely to interact with heparan sulfate proteoglycans. Since the interaction nature among FGF-2, FGF-BP2 and heparan sulfate proteoglycans is not fully characterized, it is also possible that secreted FGF-BP2 prevents the binding of FGF-2 to matrix heparan sulfate proteoglycans in the first place, and enables free FGF-2 to reach its receptors to mediate *in vitro* and *in vivo* tumor promoting activities that we observed.

It is well established that FGF-2 is a potent mitogen and required for human melanocytes in cell culture (16). In contrast to melanocytes that do not express FGF-2, abundant FGF-2 messages are detected in all melanoma tumors and most cell lines derived from metastatic melanomas (17, 18). Moreover, inhibition of FGF-2 or its receptor FGFR-1 synthesis in melanoma cell lines by antisense oligonucleotides leads to the suppression of tumor growth *in vitro* and *in vivo* (19, 20). These studies suggest that FGF-2 and its activated signal pathway play a crucial role in the development of melanoma. Interestingly, in this study we found abundant FGF-BP2 messages in all tested melanoma cell lines and melanoma tumor tissues, but not in melanocytes. Together with the evidence suggesting that FGF-BP2 is a tumor-promoting agent acting by releasing matrix bound FGF-2, it is likely that FGF-BP2 may also play a role in melanoma progression.

The clinical studies of FGF-BP2 on breast epithelia progression from normal to in situ cancer and invasive tumors suggest that there is a good correlation of FGF-BP2 with progression to a malignant phenotype. We propose that FGF-BP2 is not only a bystander but an active contributor towards malignancy. The data obtained using the MCF-7 cell model supports this hypothesis.

Methods

Cell lines and tissues. The normal neonatal epidermal melanocytes were purchased from Clonetics (Walkersville, MD), whereas 1205LU melanoma cells were obtained from Dr. M. Herlyn (Wistar Institute, PA). All other cell lines used in this study were obtained from the Lombardi Cancer Center Tissues Culture Core facility of Georgetown University.

Generation of FGF-BP2 constructs. The ORF of FGF-BP2, covering the sequence between nucleotides 132697 - 133372 in a human chromosome 4 BAC clone (GenBank accession number AC005598), was inserted into a eukaryotic pCR3.1 expression vector (Invitrogen, CA) at *Bam*HI and *Xba*I sites. In addition, the ORF of FGF-BP2 without its stop codon was generated by the polymerase chain reactions (PCR) with appropriate sense and antisense oligonucleotide primers. The amplified product was cloned into a eukaryotic pcDNA3/Myc/His expression vector (Invitrogen) at *Bam*HI and *Hind*III sites. The sequence of the insert was verified by automated cycle sequencing (ABI PRISM Dye Terminator Cycle Sequencing, Perkin-Elmer) provided by the Lombardi Cancer Center Sequencing Core facility.

Stable transfections of SW-13 and MCF-7 cells. Cells (2×10^5) at 50 - 70% confluency were transfected with 1 μ g of plasmid DNA mixed with 7 μ l of Lipofectamine in 1 ml serum-reduced Opti-MEM medium (Life Technologies, NY) for 5 h at 37 °C and 5% CO₂. The transfection medium was then replaced with fresh medium containing 10% fetal bovine serum for 24 h. Stably transfected cells were selected by culturing cells in the presence of G418 (500 μ g/ml) for 3 - 4 weeks, with a change of fresh medium every 2 days.

Soft agar growth assays. Studies of anchorage-independent growth of mock and FGF-BP2 transfected SW-13 cells in soft agar were carried out as described (13). About 10,000 - 20,000 cells in 0.8 ml of 0.35% agar (Bactoagar, Life Technologies, Inc.) were layered on top of 1 ml of a solidified 0.6% agar layer in a 35-mm dish. In some experiments, either FGF-1 or FGF-2 was included in the top layer at various concentrations (0.05, 0.10, 0.25, 0.50, 1.0 ng/ml). Colonies of 40 - 60 μ m in diameter were counted after 10 - 14 day incubation using an image analyzer. Experiments were carried out in triplicate sets of 35-mm dishes.

¹²⁵I-FGF-2 and heparin binding assays. 1 ml serum-free culture medium of either FGF-BP2/Myc/His stable SW-13 transfectants or control transfected cells was incubated with 50 ul of Ni-NTA resins (QIAGEN, Valencia, CA) in the presence or absence of 1uCi ¹²⁵I-FGF-2 (Amersham Life Science Inc.) for overnight at 4 °C. The resins were washed 3 times with phosphate buffer solution. The bound protein complexes were eluted with 100 ul of 2X Tris-glycine SDS sample buffer containing 0.1 M mercaptoethanol at 95 °C for 5 min, and the solubilized samples were subjected to autoradiography and immunoblotting analysis using mouse monoclonal antibodies specific for the Histidine or Myc tags, or custom made rabbit polyclonal antibodies specific for FGF-BP2 peptides (Research Genetics, Inc., Huntsville, AL). Under similar conditions, serum-free culture media of either FGF-BP2/Myc/His transfectants or mock control cells were incubated with 50 ul of heparin-Sepharose, and the eluted bound proteins were subjected to immunoblotting analysis.

Immunoblotting analysis. Proteins were separated by SDS-PAGE on 15% polyacrylamide gels, transferred onto nitrocellulose membranes, probed with appropriate antibodies, and the immunoreactive bands were visualized using the electrochemiluminescence detection system (ECL system, Amersham).

Tumor growth in nude mice. Mock or FGF-BP2 transfected SW-13 cells (2 X 10⁶ cells/site) were injected subcutaneously in female athymic nude mice (NCR nu/nu; Harlan Sprague-Dawley, Indianapolis, IN), with 3 animals per treatment. Mock or FGF-BP2 transfected MCF-7 cells (2 X 10⁶ cells/site) were injected into the mammary fat pad of female athymic nude mice (NCR nu/nu; Harlan Sprague-Dawley, Indianapolis, IN), with 15 animals per treatment. The animals were observed for at least 8 weeks for tumor formation. Tumor sizes were estimated from the product of the perpendicular diameters of the tumors. After the mice were sacrificed, tumors were extracted for total RNA for FGF-BP2 expression analysis.

Northern blotting analysis. Total RNAs were isolated with the RNA STAT-60 method using commercially available reagents and protocols (RNA STAT-60TM, Tel-Test, Friendswood, TX). 20 ug of total RNA were fractionated by electrophoresis in 1% formaldehyde-agarose gel and then blotted onto nylon membranes (MSI, Wesboro, MA). Premade blots with RNA from multiple human tissues were purchased from Clontech (Palo Alto, CA). The blots were prehybridized in 5 X SSC (0.75 M NaCl, 0.075M sodium citrate), 50% formamide, 1% SDS and

1X Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 20 ug/mL sonicated salmon sperm DNA) for 4 h at 42 °C. Hybridization was carried

out in the prehybridization buffer plus heat-denatured [α -³²P]dCTP-labeled cDNA probes at 42 °C for 16 h. FGF-BP1 and FGF-BP2 cDNA fragments containing ORFs were used for probes. The probes were prepared by random-primed DNA labeling (Amersham). After hybridization, blots were washed in 2 X SSC at room temperature for 5 min, followed by 2 X SSC and 1 % SDS at 50 °C for 30 min, and 0.1 X SSC at room temperature for 30 min. Autoradiography was performed using intensifying screens at -70 °C.

***In situ* hybridization.** *In situ* hybridization was performed with digoxigenin-labeled riboprobes using the procedure essentially described by Panoskaltsis-Mortari and Bucy (21) with some modifications. Briefly, the *Bam*HI - *Xba*I fragment of FGF-BP2 containing the complete coding region (684 bp) was transcribed in sense and antisense orientation using T3 and T7 polymerases, respectively with digoxigenin RNA labeling mix (Boehringer Mannheim). Mock or FGF-BP2 transfected SW-13 cells were cultured on baked slides and fixed in 10% formalin, whereas paraffin sections of melanoma tissues obtained from the Lombardi Cancer Center Histopathology and Tissue Shared Resource Core facility were incubated at 65 °C for 2 h, washed twice with xylenes for 5 min each, followed by a series of ethanol washes (100%, 90%, 80% and 70%). Prior to hybridization, cells and tissue sections were rehydrated in phosphate buffered solution, treated with proteinase K solution (10 ug/ml) for 10 min at 37 °C, incubated with 0.2 M HCl for 10 min at room temperature, acetylated in 0.25% acetic anhydride in 100 mM triethanolamine, pH 8.0 for 15 min and then rinsed with 2 X SSC. The samples were hybridized in hybridization solution (4 X SSC, 50% formamide, 1 X Denhardt's solution, 0.5 mg/mL denatured salmon sperm DNA, 0.25 mg/ml yeast tRNA, and 10% dextran sulfate) containing 1.0 ug/ml heat-denatured riboprobe for overnight at 42 °C. After hybridization, the slides were washed in 2 X SSC for 5 min, STE buffer (500 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA) for 5 min, then incubated with RNase A (10 ug/mL) at 37 °C for 30 min. The sections were sequentially washed with the following solutions at 42 °C: 2 X SSC, 50 % formamide for 30 min, 1 X SSC for 5 min and 0.5 X SSC for 5 min. The hybridized digoxigenin riboprobes were detected using alkaline phosphatase conjugated-sheep-anti-digoxigenin antibodies with BCIP/NBT (toluidinium salt/nitroblue tetrazolium) as a substrate.

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Appendices

A. Original statement of work: Role of mp170 seprase in breast cancer

Task 1. Biochemical characterization of mp170.

- A. Substrate specificity determination for secreted wild type mp170 [Months 1-18].
 - a. Construct and express secreted form of mp170.
 - b. Determine cleavage sites on potential substrates.
 - c. Explore mp170 specificity on regions N- and C-terminal to natural cleavage sites using quenched fluorescent peptide substrates.
- B. Define functional domains/residues of mp170 [Months 18-37].
 - a. Construct deletion and substitution mutant cDNAs.
 - b. Express the mutants and analyze the effects of mutation on mp170 activity and subunit dimerization.

Task 2. Effect of mp170 on breast tumor cells.

- A. mp170 overexpression in MCF-7 cells [Months 1-6].
 - a. Transfect cells with wild type mp170 cDNA and analyze stable transfectants for their growth and invasive properties.
 - b. Transfect cells with some of the mutant cDNAs generated in Task 1B and examine their effects on tumor cell growth and invasion [Months 24-36].
- B. mp170 inactivation in MDA-MB-436 cells [Months 6-24].
 - a. Construct ribozymes targeting mp170.
 - b. Establish ribozyme stably transfected MDA-MB-436 cells and analyze their growth and invasive properties.

Task 3. Effect of stromal mp170 on tumor growth/invasiveness [Months 36-48].

- A. Generate mp170 inactivated WI-38 fibroblasts.
- B. Evaluate the *in vitro* effect of stromal mp170 on the proliferation and invasiveness of tumor cells in co-culture systems.
- C. Assess the influence of stromal mp170 on tumorigenicity and metastatic abilities of ML-20 and *lacZ* transduced MDA-MB-231 cells in nude mice.

B. Amended statement of work: Role of a novel secreted FGF-binding protein (FGF-BP2) in breast cancer.

Task 1. Characterize FGF-BP2 expression in human breast cancer tissues by *in situ* hybridization.

A detailed study on the mRNA expression patterns of FGF-BP2 in normal, hyperplastic, lobular (*in situ* and *invasive*) and ductal carcinoma (*in situ* and *invasive*) breast tissues. Depending on the availability of generated antibodies against FGF-BP2, parallel Immunohistochemical studies on these samples will also be conducted.

Task 2. Characterize FGF-BP2 function in human breast cancer cells.

- A. Overexpress FGF-BP2 in MCF-7 breast cancer cells
- B. Examine tumor promoting activity of FGF-BP2 transfected breast cancer cells in *in vitro* and *in vivo* assays.

C. Curriculum Vitae

BIOGRAPHICAL SKETCH

NAME		POSITION TITLE	
Emma Bowden, Ph.D.		Research Instructor	
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training).</i>			
INSTITUTION AND LOCATION		YEAR(s)	FIELD OF STUDY
University of Kent at Canterbury Bristol University	B.Sc	1991	Biochemistry
	Ph.D.	1995	Cell and Molecular Biology

PROFESSIONAL POSITIONS:

- 1989-1991 Research Student at Glaxo Institute Molecular Biology, Geneva, Supervisor: Dr. M. Payton.
- 1991-1995 Ph.D. student in the Department of Biochemistry, Univ. Bristol, Supervisors: Dr. G. Banting and Dr. J. D. McGivan. Funded by the Medical Research Council.
- 1995-1999 Postdoctoral Fellow: Lombardi Cancer Center, Georgetown University, Supervisor: Dr. Susette C. Mueller.
- 2000-2001 Research Fellow: Lombardi Cancer Center and Department of Oncology, Georgetown University, Supervisor: Dr. Anton Wellstein
- 2001- Research Instructor: Lombardi Cancer Center and Department of Oncology, Georgetown University.

MEMBERSHIPS AND RECENT HONORS:

American Society for Cell Biologists

1st Prize in 1st Annual Lombardi Cancer Center Science Fair, Postdoctoral Category (1999)

1st Prize in 13th Annual Georgetown Student Research Days, Postdoctoral Category (1999)

PUBLICATIONS:

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